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The thesis I have reviewed describes the results of experiments performed by Matisa Alla at the Institute of Human Genetics PAS in Poznań, Poland, during her PhD studies. The research presented in the evaluated dissertation was supervised by Prof. Jadwiga Jaruzelska. Dr. Maciej Śmiałek served as an assistant supervisor of Matisa's studies.

The thesis has the form of a classical manuscript written in English, whose structure and general organization are typical for PhD theses in the field of molecular biology. Matisa's dissertation is very well written, in good and fully understandable English, and, what is worth noting, almost without editorial mistakes. The manuscript I have reviewed is, however, very long—consisting of 217 pages—which makes it the longest PhD thesis I have ever evaluated. Nevertheless, it is prepared in a reader-friendly style, mainly due to its extremely clear structure and well-organized and excellently described content. Understanding the presented information is not difficult thanks to numerous figures summarizing experimental data, as well as schematic ones that introduce the reader to the subject of Matisa's investigation. Again, the number of figures and tables included in the manuscript is enormous: 59 regular figures in the text, 19 supplementary ones, and 12 tables. Usually, I do not mention such editorial details in my reviews, but in this case, I believe that designing, performing, and analyzing the data, as well as presenting them in the dissertation, is exceptional given the amount and quality of data generated. While reading the thesis, I clearly see Matisa's determination to reach the goals of her studies and her curiosity to uncover novel molecular pathways and mechanisms. It is worth noting the wide range of different molecular and cellular methods she

used to address her research questions, including high-throughput technologies like RNA-seq and eCLIP—two modern techniques that are not trivial to perform, and even more difficult to interpret when dealing with global datasets. Here, Matisa not only carried out these complex experiments but also analyzed and interpreted the global results obtained. This broad range of techniques applied by Matisa during her PhD studies shows that she is already a mature young scientist and fully deserves a PhD degree.

I t has been well documented in the literature that NANOS proteins are conserved post-transcriptional RNA-binding regulators, crucial for embryonic germline development and maintenance across a wide range of metazoans. There are three paralogs of NANOS encoded in mammalian genomes: NANOS1, NANOS2, and NANOS3, which perform different, yet partially overlapping, functions. Matisa's project mainly focused on the impact of a mutated variant of NANOS1, p.[Pro34Thr;Ser78del], identified in infertile male patients, on early human primordial germ cell (PGC) development using a defined *in vitro* embryonic stem cell differentiation model, in which PGC formation is trackable due to the NANOS3-tdTomato reporter knock-in. It has previously been shown that this mutated NANOS1 variant impairs its interaction with the DEAD-box helicase GEMIN3, which, in consequence, leads to upregulation of mRNAs encoding pro-apoptotic proteins. Additional investigations revealed that the mutated NANOS1 variant studied here influences proliferation, cell cycle progression, and apoptosis in a seminoma germ cell line model. Therefore, the main goal of her study was to provide comprehensive insight into the structure and dynamics of the NANOS1 RNP interactome during the specification and early differentiation of human PGCs. I have no doubt that the subject is original and novel, since the NANOS1 RNA interactome has not yet been explored in human PGCs. In fact, the entire field of post-transcriptional gene regulation involving the RNP interactome in germ cell development remains largely unexplored. Thus, Matisa tackled a very interesting, but also ambitious, scientific problem in her studies.

She showed that the mutated NANOS1 variant analyzed in the thesis unexpectedly interacted with many more mRNA targets than the unmutated wild-type NANOS1. At the PGC stage, the mutated NANOS1 protein interacted with 385 targets, compared to only 5 mRNAs bound by the wild-type variant. Matisa demonstrated experimentally, combining RNA-seq with eCLIP methods, that the sites of interaction (exons, introns, 5'UTRs, 3'UTRs) also changed, making the interaction profile of the mutant much broader. She suggested that this likely induces

inappropriate repression of mRNAs important for PGC differentiation. These observations suggest a possible dominant effect of the mutated NANOS1 variant.

Since both mutations present in the NANOS1 variant analyzed affect the domain important for interaction with the deadenylation complex CNOT, one could hypothesize that the described unspecific recognition of mRNA targets results in repression of transcripts encoding proteins necessary for the transition from the premesendoderm (pre-me) stage to PGCs. Among the mRNA targets recognized by the mutated NANOS1 protein, two key transcription factors were found: OCT4 and NANOG. Given their critical roles in maintaining pluripotency and initiating germ cell-specific gene expression, Matisa has concluded that their diminished expression compromises the transition from pre-me to PGCs. However, this must be further tested in additional experiments, based on the data presented in the reviewed thesis.

Another interesting result derived from the combined eCLIP and RNA-seq data was the finding that the mutated version of NANOS1 reduces the levels of mRNAs encoding factors involved in WNT signaling. This pathway is known to be fundamental for embryonic development and, most importantly in this context, for PGC lineage specification. In short, Matisa collected solid and convincing data indicating that both pluripotency factors (e.g., MYC) and regulators of differentiation (e.g., AXIN2 and β-catenin) are downregulated in cells expressing the mutated NANOS1 variant. This indicates a significant impact of the mutated protein on WNT signaling, and, in my opinion, is the most important Matisa's finding. This effect is most likely caused by binding of the mutated NANOS1 to WNT pathway mRNAs, influencing their stability and translation, although this final conclusion requires additional direct experiments for confirmation. I would like to ask Matisa during her PhD thesis defense what kind of experiments she would propose to further validate the connection between mRNA degradation and WNT pathway activity. She also observed that the expression level of EOMES, a known PGC competence marker and WNT signaling target, was downregulated upon overexpression of the mutated NANOS1. As she concluded, this reduction likely compromises specification and subsequent differentiation of PGCs *in vitro*. I fully agree with her interpretation.

In addition to its effects on the WNT pathway, the overexpression of the mutated NANOS1 also induced coordinated dysregulation of other proteins—namely THBS1, CDH1, and VCL—

which parallels processes reminiscent of epithelial-to-mesenchymal transition (EMT) observed in cancer, where destabilization of cell-cell junctions occurs. Matisa hypothesized that the mutated NANOS1 triggers EMT-like processes, disrupting PGC identity and pushing cells toward a somatic-like lineage. I found this hypothesis very interesting and would suggest pursuing this direction in future research. However, it should be noted that these results appear preliminary and raise more questions than they answer, particularly regarding the underlying mechanisms.

The data presented in the thesis also showed that the overexpression of mutated NANOS1, through the aberrant repression of WNT, TGF-β, and pluripotency factors, leads to the downregulation of several critical PGC markers. Two of them are likely regulated directly by the mutated NANOS1, while most appear to be affected indirectly. As these markers are essential for human PGC fate, changes in their expression levels may influence germ cell programming, as suggested and described in detail in the dissertation. Additionally, Matisa showed that WNT5A, a signaling protein crucial for PGC migration and proliferation, is downregulated in cells overexpressing the mutated NANOS1. This may further impair PGC migration.

Overexpression of the mutated NANOS1 variant also resulted in a reduced number of PGCs, which Matisa correlated with downregulation of NANOS3 mRNA. She linked this observation to *in vivo* data from patients carrying this NANOS1 mutation, in whom germline cells are absent from the testes.

In her final experiment, Matisa attempted to rescue the phenotype of PGCs overexpressing the mutated NANOS1 by inhibiting the WNT pathway with Wnt-C59. The results showed increased levels of pluripotency markers (OCT4, NANOG), as well as NANOS3 and PRDM14. Interestingly, Wnt-C59 inhibition also reduced the expression of BMP4, a protein known to promote mesoderm formation and limit PGC numbers after specification. Halting prolonged BMP signaling at the end of PGC specification seems to restore normal expression of germ cell markers. In contrast, blocking WNT signaling with Wnt-C59 appears to partially reverse mesodermal differentiation. Moreover, treatment of PGCs with the Wnt inhibitor in the presence of the mutated NANOS1 restored PGC identity, pluripotency, and germ cell marker

expression. This is a very interesting and promising result, opening new directions for potential therapeutic applications.

To conclude, Matisa Alla's thesis presents novel and original results from numerous well-designed experiments. The data were properly analyzed and thoroughly discussed in relation to the current literature. The discussion demonstrates that Matisa has a deep understanding of the field and can critically analyze her own results. The final conclusions are well-supported. It is also worth highlighting the extensive range of molecular and cellular methods used in this study. Matisa dedicated a great deal of time to developing and characterizing new cell lines, which were later used for transcriptome and interactome analyses. I also highly appreciate the use of advanced computational tools in the bioinformatic analyses. Her study reveals how the mutated NANOS1 variant (p.[Pro34Thr; Ser78del]) disturbs transcriptomic and signaling networks essential for early germ cell specification. This knowledge may provide important mechanistic insight into male infertility in patients carrying these mutations. Matisa Alla's doctoral thesis meets all the criteria set out in the Act (art. 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce). Therefore, I recommend that the Scientific Board of the Institute of Human Genetics proceed with all necessary steps to award Matisa Alla the PhD degree. Due to the originality and significance of the scientific findings presented, I also recommend that the thesis be considered for a special distinction.

Signed by /
Podpisano przez:

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